Monomeric fluorescent timers that change color from blue to red report on cellular trafficking

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Supplementary Table 2. Kinetic rate constants for transitions between the FT chromophore states during its maturation at 25^0C in S2 cells and 37^0C in HeLa cells.

Supplementary Methods.

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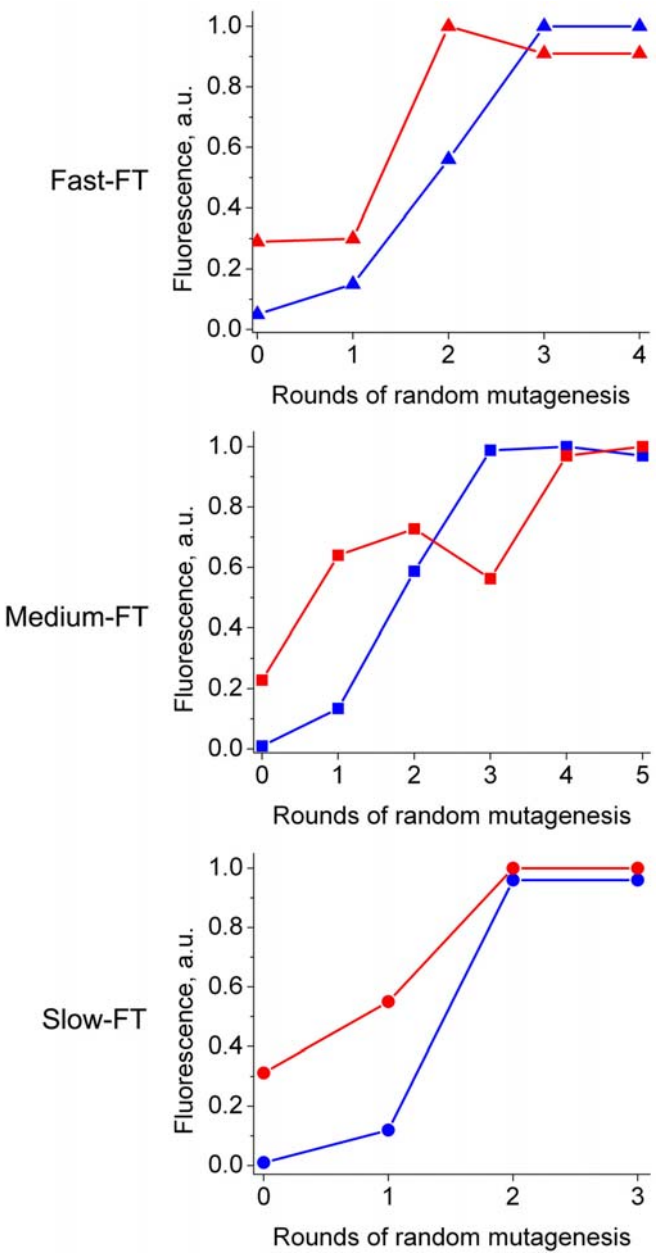
Supplementary Figure 1. Evolution of fluorescence intensity of the blue and red forms of the best intermediate variants in each round of mutagenesis during screening for Fast-FT, Medium-FT and Slow-FT.



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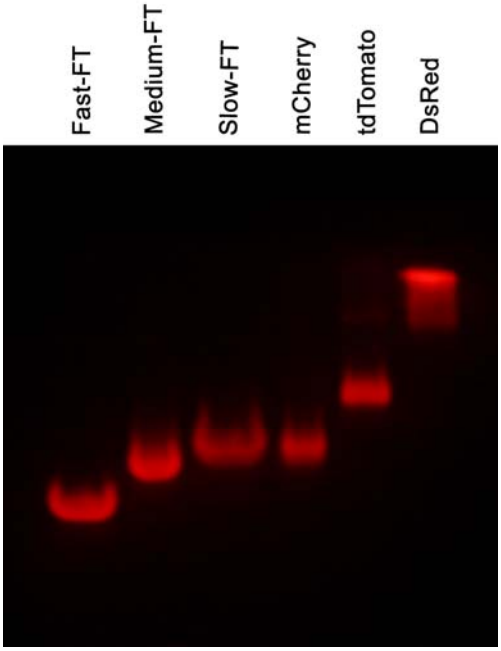
Supplementary Figure 2. Semi-native polyacrylamide gel with purified DsRed, tdTomato, mCherry and the matured red forms of Fast-FT, Medium-FT, and Slow-FT.



Aliquots of 10 μg of purified fluorescent proteins were applied in 10 μl aliquots to semi-native 15% polyacrylamide gel. The gel was photographed using Leica MZ16FL fluorescence stereomicroscope equipped with 570/30 nm excitation and 605/30 nm emission filters. DsRed, tdTomato and mCherry were used as the tetrameric, dimeric and monomeric native protein standards, respectively.

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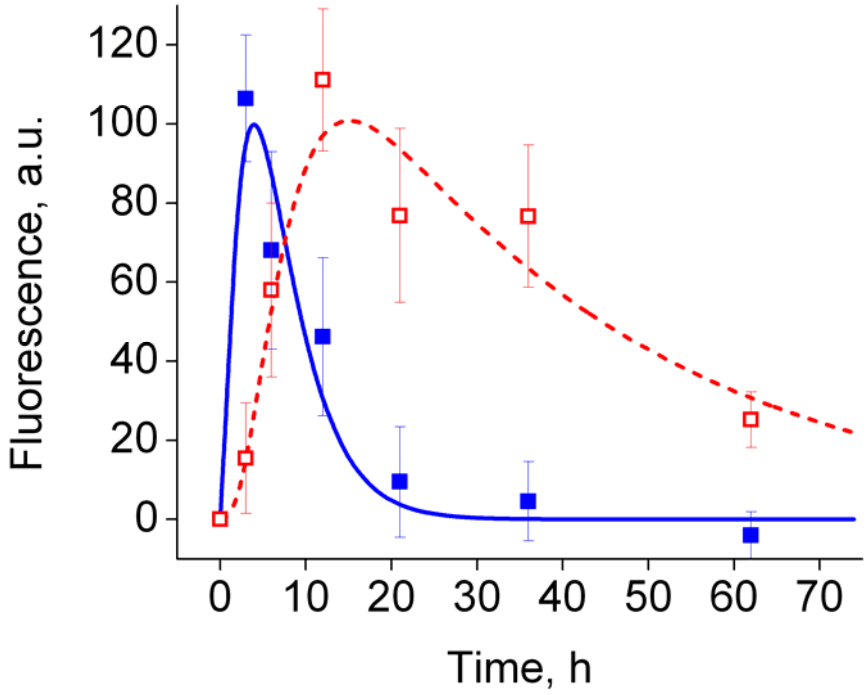
Supplementary Figure 3. Behavior of the Medium-FT expressed in the cytoplasm of HeLa Tet-Off cells.



Change of the blue (closed squares) and red (open squares) fluorescence emission of the HeLa Tet-Off cells cytoplasmically expressing the Medium-FT with time detected using flow cytometry. Time 'zero' corresponds to the addition of doxycycline at 12 hours after cell transfection. The experimental points were fitted with the blue solid line for the blue form and with the red dotted line for the red form using kinetic scheme shown in Fig. 4. The coefficients of determination, R^2, are larger than 0.90.

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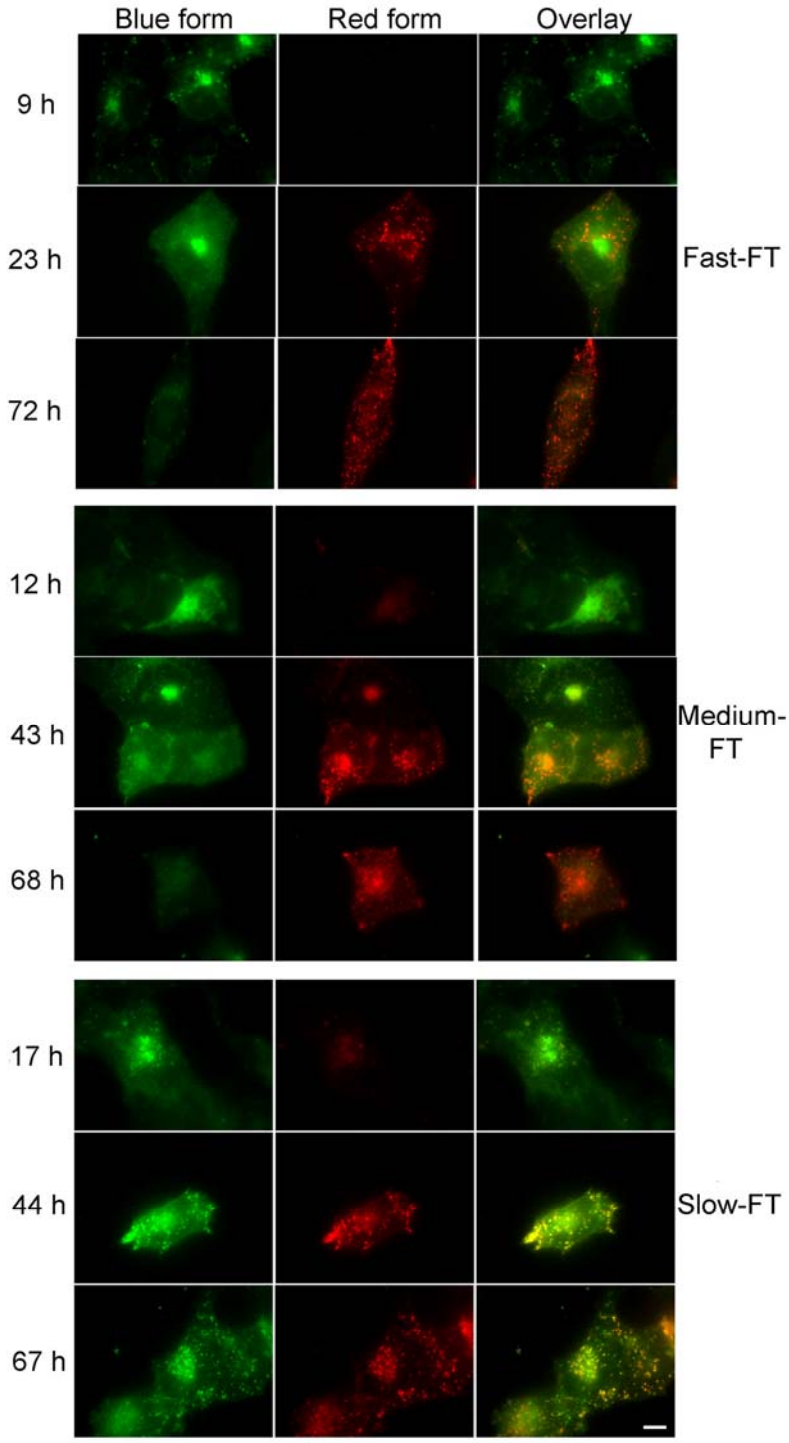
Supplementary Figure 4. Time dependence of localization of the fusion proteins of LAMP-2A with Fast-FT, Medium-FT and Slow-FT in live Cos-1 cells.



The blue and red forms of LAMP-2A-FTs are shown in green and red pseudocolors, respectively. The indicated times correspond to the times after the addition of doxycycline. Bar is 10 μm.

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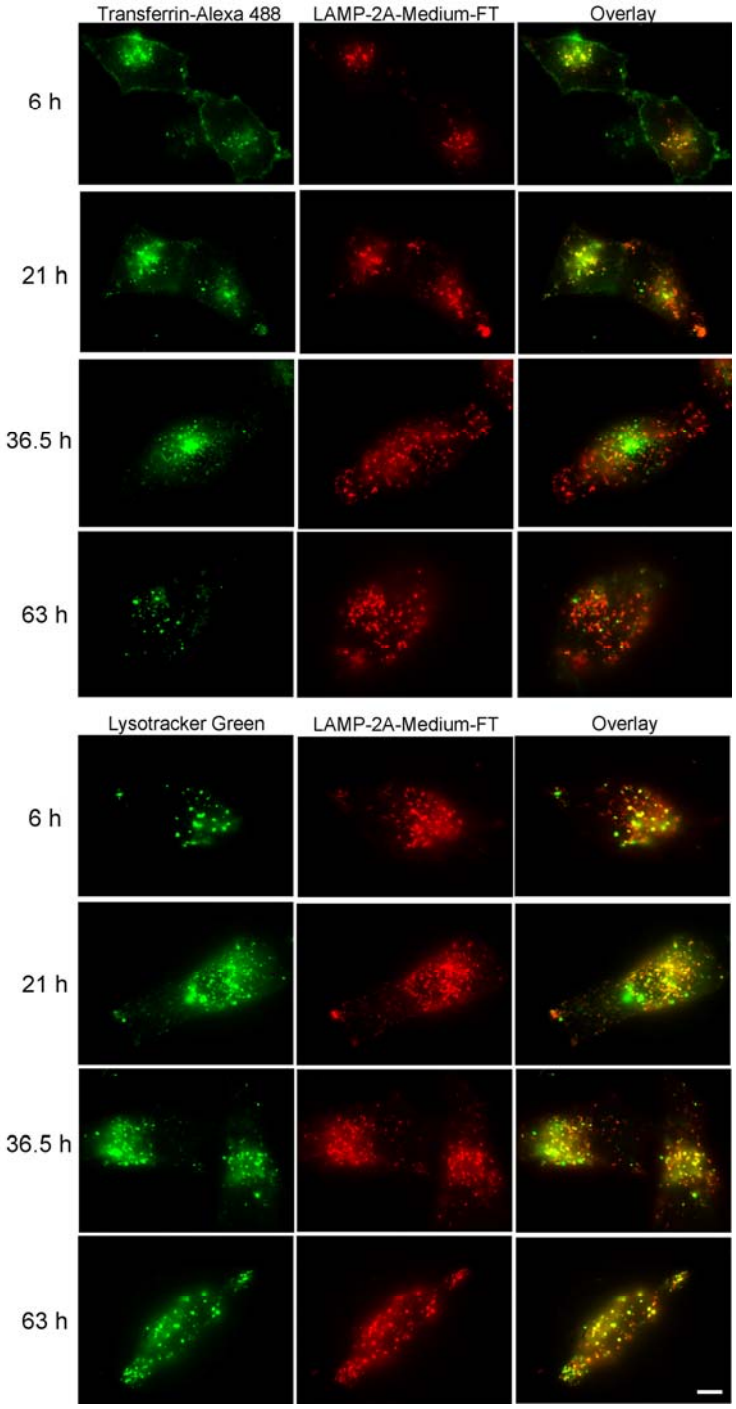
Supplementary Figure 5. Visualization of early and recycling endosomes, late endosomes and lysosomes in live HeLa Tet-Off cells transfected with LAMP-2A-Medium-FT.



The transferrin-Alexa 488 and Lysotracker Green are shown in green, and the red form of the LAMP-2A-Medium-FT is shown in red. The indicated times correspond to the times after the addition of doxycycline. To emphasize that a part of the red LAMP-2A-Medium-FT merged with transferrin-Alexa 488 and Lysotracker Green we have enhanced the FT signal. These data were not used for calculations. Bar is 10 μm.

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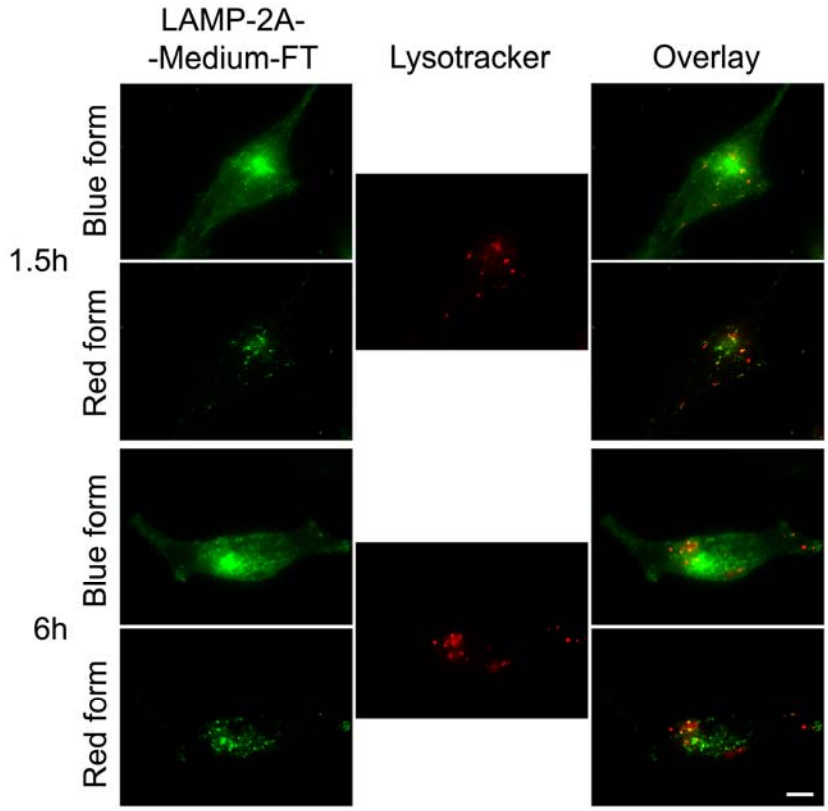
Supplementary Figure 6. HeLa cells expressing LAMP-2A-Medium-FT at 1 and 6 hours after inhibition of the protein expression co-stained with Lysotracker Green.



The blue and red forms of LAMP-2A-Medium-FT are shown in green pseudocolor, and Lysotracker Green is shown in red pseudocolor. The indicated times correspond to the times after the addition of doxycycline. To emphasize that a part of the red LAMP-2A-Medium-FT merged with the Lysotracker Green we have enhanced the FT signal. These data were not used for calculations. Bar is 10 μm.

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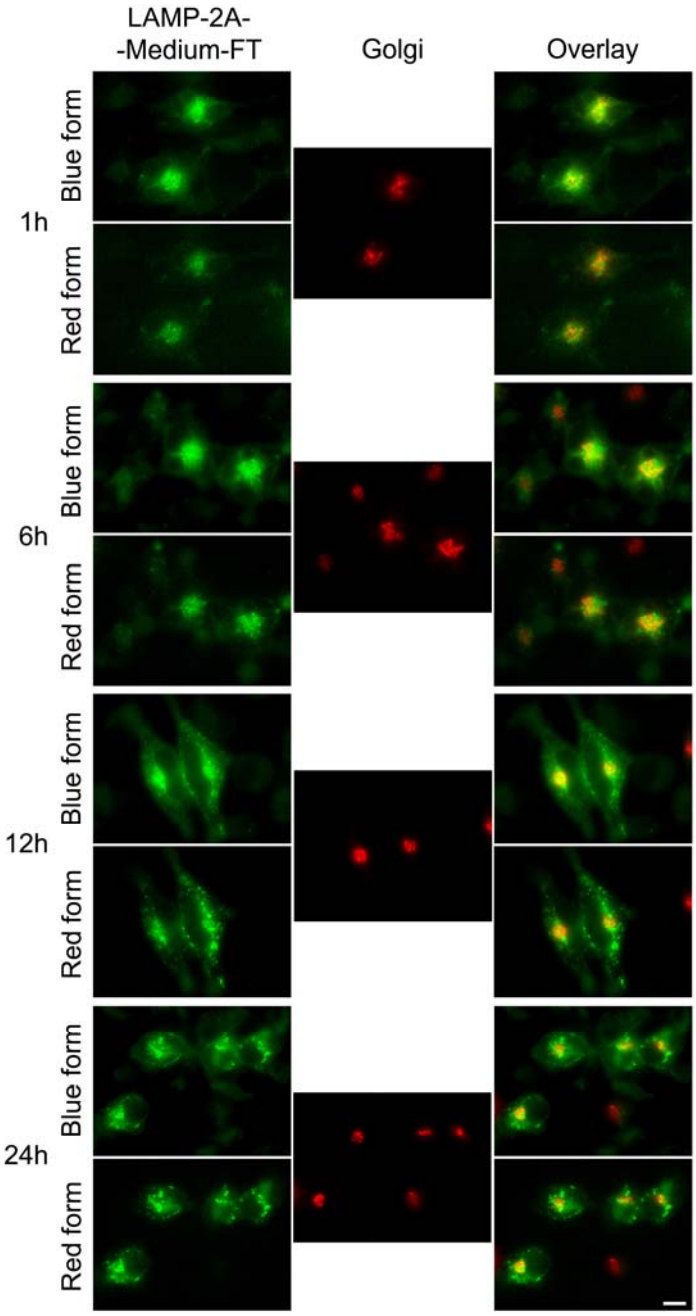
Supplementary Figure 7. HeLa cells expressing LAMP-2A-Medium-FT co-stained with the Golgi network marker, β−1,4-galactosyltransferase-EYFP.



The β−1,4-galactosyltransferase (GalT) is a membrane-bound enzyme localized predominantly to the trans-Golgi network[1-3]. The blue and red forms of LAMP-2A-Medium-FT are shown in green pseudocolor, and GalT-EYFP is shown in red pseudocolor. The indicated times correspond to the times after the addition of doxycycline. To emphasize that the red LAMP-2A-Medium-FT did not merge with the GalT-EYFP we have enhanced the red FT signal. These data were not used for calculations. Bar is 10 μm.

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Supplementary Table 1. Mutations found during the molecular evolution of mCherry into the Fast-FT, Medium-FT and Slow-FT.

Mutagenesis	Fast-FT		Medium-FT		Slow-FT	
	new key mutations found	**mutations observed but not selected**	**new key mutations found**	**mutations observed but not selected**	**new key mutations found**	**mutations observed but not selected**
Original template	mCherry					
Site-specific at: 42, 44, 65, 69, 106, 148, 203, 224	+ K69R/A224S	-	+ K69R	L44M, V106A,I,L, S148T,F, I203Y,T,L, A224G,C	+ K69R	M65L, S148F,I, I203Y,T,L
Random round #1	+ M18V/L84W/ /S149T	-	+ M18L/L84W	G23D, A76V, G159C, G175D, V201I, R227C	+ M18V/G24D/ L84W/A179V/ /Q110H	H17Y, E30V, T43S, F63C, S112C, A147T, K182M, V193L, L205M, Y221C
Random round #2	+ S112T/D178N	-	+ L205M	H17N, G24S, E39D, A56T, T107I, T128I, M143K, T184I, K186M	+ E32V/D178N//I120V	E26K, T43N, R126C, H176D, Q194L, S209Y
Random round #3	+ H17Y/E34K/ /S149I/N202D/ /S209T	T184S	+ Q194L/T43S/ /H176D/Y221C	K10N, S132Y, H176Q, K182R, Q194L	no new mutations	-
Random round #4	no new mutations	-	+ M152I/R227H	N23D, G24D, T128S, S132F, M152L	not performed	-
Random round #5	not performed	-	no new mutations	-	not performed	-
Site-specific at: 69, 84, 143, 179, 203, 205	no new mutations			W84Y, M143L, L205M	no new mutations	M143I

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Supplementary Table 2. Kinetic rate constants for transitions between the FT chromophore states during its maturation at 25^0C in S2 cells and 37^0C in HeLa cells.

Protein	Temperature	k_C, h^{-1}	k_B, h^{-1}	k_I, h^{-1}	k_R, h^{-1}	k_D, h^{-1}
Fast-FT	25^0C	$1.3x10^{-2}$	$3.8x10^{-1}$	$3.0x10^{-1}$	$4.9x10^{-2}$	$2.0x10^{-3}$
Medium-FT		$1.0x10^{-2}$	$4.8x10^{-1}$	$1.7x10^{-1}$	$2.5x10^{-2}$	$5.0x10^{-3}$
Slow-FT		$9.0x10^{-3}$	$2.0x10^{-2}$	$1.4x10^{-1}$	$4.0x10^{-2}$	n.d.
Fast-FT	37^0C	n.d.	8.7	$7.8x10^{-1}$	$1.4x10^{-1}$	n.d.
Medium-FT		$2.9x10^{-1}$	2.2	$3.1x10^{-1}$	1.2	$2.8x10^{-2}$
Slow-FT		$3.0x10^{-1}$	$4.8\ x10^{-2}$	$1.9x10^{-1}$	$1.4x10^{-1}$	$4.8x10^{-2}$

Values of the kinetic constants are a result of fitting of the experimental data with a kinetic model described in the Main text performed with Gepasi chemical kinetics simulation package[4]. Note that k_C and k_D rate constants were not determined for the Fast-FT at 37^0C and k_D for the Slow-FT at 25^0C because of the limited number of data points available for these conditions.

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Supplementary Methods

Library screening. LMG194 cells were grown at 37^0C overnight in RM minimal medium supplemented with Ampicillin. The protein expression was induced with 0.2% arabinose for 1 (for screening of Fast-FT) or 4 h (for screening of Medium-FT and Slow-FT). Then Fast-FT and Medium-FT libraries were screened using MoFlo (Dako) fluorescence activated cell sorter (FACS). In the case of Slow-FT library, the cells were pelletted down, resuspended in RM medium supplemented with glucose, and FACS sorted 20 h later. For FACS sorting, the bacteria were washed with Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) and then diluted with PBS to optical density of 0.02 at 600 nm. MoFlo cell sorter was equipped with Krypton, Argon, and Argon-Krypton mixed-gas lasers. For detection of the blue (450/65 nm filter), green (530/40 nm filter) and red (580 nm longpass filter) fluorescence emissions, 407, 488 and 568 nm laser lines were used, respectively. Typically about 10 sizes of each library were screened. The collected brightest blue/non-green/non-red bacterial cells were rescued in rich Super Optimal broth with Catabolite repression (SOC) medium at 37^0C for several hours, and then were spread on 0.45 μm nitrocellulose membranes placed on Petri dishes with Luria-Bertani medium (LB)/Ampicillin and incubated for overnight at 37^0C. Next morning the membranes were placed on Rich Medium (RM)/Ampicillin Petri dishes with 0.2% arabinose to induce protein expression. After incubation for 1 h (Fast-FT) or 4 h (Medium-FT) at 37^0C, the brightest blue/non-green/non-red colonies were marked off using Leica MZ16F fluorescence stereomicroscope equipped with custom blue (390/40 nm exciter, 460/40 nm emitter), green (480/40 nm exciter, 530/40 nm emitter), and red (570/30 nm exciter, 615/40 nm emitter) filter sets (Chroma). In the case of Slow-FT, additional 20 h of protein maturation time were required to select the brightest blue/non-green/dim-red colonies. 1 h (Fast-FT) or 4 h (Medium-FT and Slow-FT) after the start of the expression, the membranes were placed on Petri dishes with RM/Ampicillin/0.2% glucose to repress the protein production. Then, 24 h (Fast-FT or Medium-FT) or 48 h (Slow-FT) later, the brightest red/non-blue/non-green colonies among the previously marked clones were selected.

Protein purification. To purify recombinant proteins, LMG194 cells grown overnight were diluted to optical density 1.0 at 600 nm, and 0.2% arabinose was added for induction of protein

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expression. The bacterial cultures were then grown at 37^0C in 50 ml tubes filled to the brim and tightly sealed to restrict oxygen supply. After 1 h, the cultures were pelletted down in the same tightly closed tubes. After opening the tubes, the proteins were purified using B-Per reagent (Pierce) and Ni-NTA resin (Qiagen) within 15 min with all procedures and buffers at 4^0C.

Insect and mammalian plasmid construction. To make pRmHa-3-FTs plasmids, the PCR amplified *Eco*RI-*Bam*HI fragments encoding FTs were inserted into pRmHa-3 vector [21]. To generate LAMP-2A-FTs fusion proteins, the PCR-amplified *Xho*I-*Age*I fragments encoding FTs were swapped with TSapphire-GFP in the pcDNA-3.1-LAMP-2A-TSapphire-GFP vector (gift of Dr. D.Reeves). To make pTRE-FTs or pTRE-LAMP-2A-FTs plasmids, the PCR amplified *Eco*RI-*Xba*I fragments encoding either FTs or LAMP-2A-FTs fusion proteins, respectively, were inserted into pTRE vector (Clontech).

Transfection. Transfection of Drosophila S2 cells and all mammalian cells was carried out with the Effectene reagent (Qiagen). For microscopy imaging, HeLa, Cos-1, NIH3T3 or HeLa Tet-Off cells were plated on 25 mm glass coverslips in Dulbecco modified essential medium (DMEM) (Invitrogen) supplied with 10% Fetal Bovine Serum (FBS) (Sigma). S2 cells were cultured in Schnieder's medium (Gibco) supplied with L-glutamine and 10% FBS (Sigma) at 25^0C. To establish stable pre-clonal cell cultures, pRmHa-3-FTs plasmids were co-transfected with pCoBlast vector (Invitrogen) and selected with 25 μg/ml of blasticidin for 2 weeks.

Staining of cellular compartments in mammalian cells. For staining of early and recycling endosomes with transferrin-Alexa 488, cells were washed with PBS, and incubated in DMEM without FBS for 30 min at 37^0C. After adding 50 μg/ml of transferrin-Alexa 488, cells were incubated at 4^0C for 15 min before rinsing with PBS and further incubation in DMEM supplied with 10% FBS for the next 20 min at 37^0C. For staining of late endosomes and lysosomes, cells were incubated with 100 nM of Lysotracker Green for 30 min at 37^oC before imaging. For visualization of Golgi network, HeLa Tet-Off cells were co-transfected with pTRE-LAMP-2A-Medium-FT and pGalT-EYFP using 10:1 plasmid ratio.

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